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(54) Title: USE OF IGF-I OR ANALOGUES THEREOF IN THE PREVENTION OF DIABETES

(57) Abstract

The invention relates to the use of IGF-I or analogues thereof in the manufacture of a medicament useful in the prevention of diabetes, in delaying the clinical onset of diabetes and with protective effect against diabetes. It also relates to the use of IGF-I or analogues thereof in the manufacture of a medicament preventing beta cell destruction and the regulation of T cells in subjects which are at high risk of development of diabetes. The invention also relates to the method for protecting against diabetes, prevention of diabetes and delaying the clinical onset of diabetes by administration of IGF-I or analogues thereof.

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USE OF IGF-I OR ANALOGUES THEREOF IN THE PREVENTION OF DIABETES.

The present invention relates to the use of IGF-I or analogues thereof in the manufacture of a medicament useful in the prevention and delaying the clinical onset of diabetes and having a protective effect against diabetes.
The medicament is also useful in preventing beta cell destruction and regulating of T cells.

SUMMARY

Insulin like growth factor-1 (IGF-1) and insulin are structural homologues, and elicit insulin-like and growth-promoting effects. In addition, IGF-1 has important effects on thymocyte replication and function independently of those of insulin. To evaluate the effect of IGF-1 on the autoimmune process of beta cell destruction, permissive recipients were adoptively transferred with T cells from diabetic donors and 10µg of rhIGF-1 were administered subcutaneously twice daily. The recipients of $7x10^6$ autoreactive T cells were followed for clinical manifestations of diabetes and examined for in situ lesions after three weeks of treatment. We observed that the administration of rhIGF-1 delays the clinical onset of the disease and reduces the final incidence of successful transfers since diabetes was observed in only 6/24 (25%) vs 12/21 (57%) in control mice. These effects were associated with a marked reduction of insulitis. Mice treated with rhIGF-1 had a higher percentage of intact islets (48.6±12% vs 1.6±1.1%, p=0.001) and a lower percentage of infiltrated islets. However, some mice developed diabetes despite rhIGF-1 administration with severely infiltrated islets, indicating thus that committed T cells were still able to invade the islets and cause beta cell destruction. Three weeks after sub-lethal irradiation and T cell inoculation no difference was noticed in the percentages of $CD4^+$ and $CD8^+$ T cells in the spleen of experimental mice. To further elucidate whether rhIGF-1 could influence the homing of committed T cells, we adoptively transferred congenic NOD-N Thy-1,1 mice with T cells from diabetic NOD Thy-1,2 mice and monitored the numbers of Thy-$1,2^+$ T cells present in lymphoid organs after three weeks of treatment. The administration of rhIGF-I was found to reduce significantly the percentage of Thy-$1,2^+$ T cells in the spleen (10.8±1.3% vs 17.2±3.9%, p=0.004) in contrast to the thymus (68.4±7.9% vs 72.87±6.2, p=0,306). The findings that rhIGF-1 has protective effects and may

act prior to islet cell invasion opens new perspectives for future experiments and preventive strategies in human type 1 diabetes.

INTRODUCTION

The non-obese diabetes (NOD) mouse is an experimental model of spontaneous diabetes resembling human type 1 (insulin-dependent) diabetes, which results from the progressive islet invasion and beta cell destruction by autoreactive T cells (1,2). This spontaneous diabetes model offers a unique opportunity of studying the autoreactive T cells involved in the process of beta cell destruction and of settling preventive strategies before clinical onset of the disease. The number of committed T cells in the spleens of diabetic animals (3) and the respective contribution of T cell subsets (4) can be evaluated in vivo during adoptive T cell transfer into non diabetic syngeneic animals.

Insulin like growth factor-1 (IGF-1), a 70-amino acid peptide structurally related to insulin, is normally considered to be a metabolic hormone which mediates many effects of growth hormone. Prophylactic insulin treatment of NOD mice during the prediabetic phase (5) as well as insulin treatment of the NOD recipients of autoreactive T cells during adult T cell transfer (6) have been shown to prevent and/or delay the onset of diabetes and to reduce the severity of insulitis. Similar results have been also obtained in BB rats (7,8), which as another animal model of spontaneous autoimmune diabetes. Since insulin is a major antigenic component of the beta cells, it was not clear from these experiments whether insulin protective effects were explained by an antigen-specific unresponsiveness of the immune system, by a direct suppressive effect on T cell function, or by a direct effect on the beta cells.

The present study was undertaken to examine whether rhIGF-1 may have protective effects in the autoimmune diabetes of NOD mice using adoptive T cell transfer experiments.

THE INVENTION

The invention relates to the use of IGF-I or analogues thereof in the manufacture of a medicament useful in the prevention of diabetes and in delaying the clinical onset of diabetes.

IGF-I has also shown to have a protective effect against diabetes, is preventing beta cell destruction in subjects which are at high risk of development of

diabetes and in the regulation of T cells in subjects which are at high risk of developing diabetes.

The invention relates to a method for treating patients having the above mentioned problems by administration of IGF-I or analogues thereof.

Possible daily dosages of IGF-I are 20 to 500 µg/kg or preferably 20 to 250 µg/kg or more preferably 100 to 200 µg/kg.

5 figures are illustrating the results of the experiments.

Figure 1	Cumulative incidence of diabetes in four independent in mice
Figure 2	Severity of insulitis and destructive lesions
Figure 3	Immunodetection of Thy-1,2$^+$ T cells in the islets of congenic NOD-N Thy-1,1 mice
Figure 4	FACS analysis of Thy-1,2$^+$ T cells within the spleen of a congenic NOD-N Thy-1,1 mouse
Figure 5	FACS analysis of Thy-1,2$^+$ T cells within the thymus of a congenic NOD-N Thy-1,1 mouse

MICE AND METHODS

1. Mice

NOD mice were bred under standard conditions in our own facilities. The incidence of spontaneous diabetes in our colony reached 80 % in females by 30 weeks whereas diabetes occurred in only 20 % of males in the same period. Congenic NOD-N Thy-1,1 mice initiated from a cross between NOD/Lt and a diabetes resistant strain NON/Lt were obtained from Ed. Leiter, Bar Harbor Michigan (10). Diagnosis of diabetes was characterized by polydipsia, weight loss, glycosuria (Urine chemstrips, Ames-Bayer, Germany) and persistent hyperglycemia (Blood glucose chemstrips, Lifescan USA). Diabetic NOD females served as donors of autoreactive T cells. Four different experiments of adoptive cell transfer of diabetes were performed using 46 male recipients and 22 diabetic females.

2. Cells

Splenocytes from diabetic mice were isolated in Hanks' balanced salt solution (HBSS) and enriched T cell populations were obtained by filtration through nylon wool columns eluting 20 to 25 % of the initial cell preparation. More than 90 % of the final cell suspension was from the Thy $1,2^+$ phenotype during flow cytometry analysis. After numeration and viability evaluation, 7.10^6 T cells were i.v. injected into 8 to 10 week-old irradiated NOD males (750 rads) according to the method of Wicker et al (3).

3. Protocol of treatment

Fast-acting insulin stock solution (Actrapid HM, Novo Nordisk Copenhagen, Denmark) was prepared with a 9 ‰ NaCl solution at a final concentration of 5 U/ml. Recombinant human IGF-1 (rhlGF-1) was obtained from Dr Anna Skottner (Kabi Pharmacia, Stockholm, Sweden) and aliquoted to a final concentration of 100 µg/ml. The day following adoptive cell transfer, mice were s.c. injected twice daily 100µl containing either 0.5 U of insulin, 10µg of rhlGF-1 or saline. Recipient mice received approximately 30 U/kg/day of insulin and 0.6 mg/kg/day of rhlGF-1 over a period of three weeks. The onset of glycosuria was monitored daily starting at day 15.

4. Histologic procedures

All mice were killed by cervical dislocation. Pancreatic glands were excised and processed for conventional histological studies after fixation in Bouin's alcoholic solution. Five µm sections were stained with haematoxylin-eosin, as described previously (6). The severity of insulitis was scored for at least 25 islets for each specimen, wherein islet cells which had no visible sign of inflammation were scored 0, islets which had lymphocytes at the periphery i.e. peri-insulitis were scored 1, islets which were mildly infiltrated (<40 %) were scored 2, islets which were completely infiltrated were scored 3. The percentages of islets of each category were compared between the different groups of mice. The number of beta cells was determined by immunohistochemistry on fixed sections, using an anti-human insulin monoclonal antibody (Novoclone HUI 018, NovoBiolabs, Bagsvaerd Denmark) diluted 1:50 and an anti-human proinsulin monoclonal antibody (Novoclone HPUI). An FITC rabbit anti-mouse IgG (Dako, Burlingame USA) dilution 1:50 was used as a conjugate.

5. T cell subset analysis

After 3 weeks of treatment, spleens from experimental animals were subjected to T cell subset analysis using an anti-Thy1,2 (clone 30H12), anti-L3T4 (clone GK 1,5) and anti-Lyt2 (clone 53-67) rat monoclonal antibodies and a FITC-conjugated anti- rat IgG kappa antibody (MARK-1, Biosys, Compiègne France). To evaluate the influence of rhlGF-1 treatment upon the homing of autoreactive T cells, the percentages of Thy-1,2$^+$ T cells injected into NOD-N Thy-1,1 recipients were determined in lymphoid organs by FACS analysis as well as in islet infiltrates by immunohistochemical procedures on pancreatic sections.

6. mRNA studies

In order to study the number of mRNA transcripts for insulin in the pancreas of experimental mice, the total RNA content was precipitated in 4M guanidine thiocyanate and then in 7.5M guanidinium hydrochloride (Sigma, St-Louis MO) solutions and extracted in chloroform-butanol (100/24, vol/vol). Four different concentrations of ARN ranging from 2.5 to 20µg were hybridized on nylon membranes with P32-labelled cDNA rat proinsulin probes(obtained from C. Dagorn, Marseille France). Films were analyzed by densitometric scanning after 24 hrs of exposure period.

7. Statistical analysis

The effects of treatment on diabetes transfer were analyzed using the Wilcoxon test. Scores of insulitis were compared using Student't test for unpaired samples.

RESULTS

Effects of rhlGF-1 treatment on T cell transfer of diabetes

In order to evaluate the effects of hormonal treatment on the diabetes transfer capacity of autoreactive T cells, we initiated the injection protocol on the day following the adoptive T cell transfer and continued for an overall period of three weeks. The effects of rhlGF-1 on blood glucose levels were determined in a separate experiment. Glucose levels dropped significantly (81.5±0.7 mg/dl) 30 minutes after a single s.c. injection of 10 µg of rhlGF-1, and after 2 hours increased above normal values (181±19.8 mg/dl) before returning to baseline. During the treatment period, the effects of rhlGF-1 on body weight were

monitored every two days. rhlGF-1 was able to maintain the body weight of recipient mice, in contrast to saline and/or insulin injections, (Table I). However, these effects were closely dependent on the presence of clinical diabetes.

The occurence of clinical diabetes was determined in rhlGF-1 treated mice and compared with saline and insulin treated groups. Diabetes was detected in only 6 out of 24 (25 %) mice treated with rhlGF-1, in contrast to 12/21 (67 %) in control mice and 6/14 (42.8 %) mice treated with insulin. Insulin like growth factor-1 was associated with a significant reduction in diabetes incidence (p=0.016) as shown in Figure 1 with a significant delay in the clinical onset of the disease. In addition, insulin-treated mice (p-0.01) also had a significantly lower incidence rate of diabetes than control mice. The treatment of diabetic NOD females with rhlGF-1 twice daily over a period of 7 days prior to transfer, failed to modify the number and degree of activation of the autoreactive T cells contained in the spleens of experimental animals and the diabetes incidence curves were found to be the same after one month.

Histological studies

The severity of insulitis was quantified and a comparison was made between the different experimental groups of animals. As shown in figure 2, when compared to sham-injected mice, mice that had been treated with rhlGF-1 were found to have a higher percentage of normal (e.g. non infiltrated) islets (48.6±12.1% vs 1.62±1.1%, p=0.001) and a lower percentage of islets with mild (15.8±5.1% vs 31.5±2.8%, p=0.016) or severe insulitis (22.43±8.8% vs 59.82±6.5%, p=0.003). However, no significative difference was found in the percentage of peri-insulitis (7±4.5 % vs 13.1±5.8%, p=0.424).

Interestingly, islets from 4/12 mice which received rhlGF-1 were free from lymphocytic infiltration in contrast to 0/11 mice in the control group. Thus, rhlGF-1 reduced both the intensity and the prevalence of insulitis.

Effects of rhlGF-1 on insulin synthesis

Although the number of intact beta cells was higher in rhlGF-1 treated animals, no difference was noticed in the intensity of the fluorescent pattern of the remaining beta cell at the end of the treatment period in mice that had been treated with either rhlGF-1 or with saline. In addition, the levels of mRNA transcripts for proinsulin in non diabetic mice during dot blot analysis were comparable in both situations, thus indicating that at the doses used in the

present experiments rhlGF-1 does not modulate significantly the rate of insulin synthesis.

Effects of rhlGF-1 on T cell homing

Because insulitis is a T cell phenomenon, we suspected that rhlGF-1 might interfere with the kinetics of the migration of committed T cells to the pancreas. Congenic NOD-N Thy-1,1 males were adoptively transferred with T cells from diabetic NOD Thy-1,2 animals. Diabetes occurred in 3/6 mice that had been treated with saline and 0/6 mice that had been treated with rhlGF-1, after 3 weeks of treatment. This apparent protective effect was also associated with a decrease in the severity of islet cell infiltrates, which were composed exclusively by T cells from donor origin with no recruitment of host T cells as shown in figure 3. When analyzed in individual mice, the number of Thy-1,2$^+$ T cells was found to be significantly lower in the spleen of treated mice with rhlGF-1 in comparison with control mice (Table III and figure 4), although no significative difference was noticed within the thymus (figure 5).

DISCUSSION

The adoptive T cell transfer model in the NOD mouse explores in vivo the capacity of autoreactive T cells to cause destructive lesions and ultimately type I diabetes. In the present study, we have demonstrated that rhlGF-1 is able to reduce the capacity of large amounts of committed T cells from invading NOD islets during adoptive T cell transfer. These results reproduce those previously obtained with human insulin (6). However, the present experiments clearly demonstrate that rhlGF-1 is more potent than insulin in preventing diabetes transfer at concentrations 10 times less to those giving comparable metabolic effects in diabetic rats (11). Despite the injection of high numbers of autoreactive T cells, rhlGF-1 was found to delay the time of onset and to reduce the maximal frequency of clinical diabetes. In addition, strong histological evidence indicate that rhlGF-1 prevents massive islet cell invasion and fully protects one third of the treated mice.

There are distinct classes of mechanisms which may be responsible for the prevention of beta cell destruction by IGF-1. First, the effect may be on the beta cells. Specific receptors on the surface of beta cells as well as local production of this growth factor have been identified (12). Recently, an enhanced IGF-1 gene expression has been shown in regenerating rat pancreas after partial

pancreatectomy (13,14). However, we were unable to find any difference in the number of insulin and/or proinsulin positive cells within the islets. However, the conservation of insulin-producing beta cells was associated with a marked reduction in islet infiltration, suggesting that the contribution of beta cell regeneration was not essential. Insulin-like growth factor 1 may also on the other hand, be considered as a regulator of insulin release in view of its inhibitory effects at physiological concentrations (15). Although the hypothesis of beta cell rest formulated during early and prolonged insulin therapy (5) might also be evacuated, no difference was noticed in the intensity of insulin staining of the beta cells and in the number of mRNA transcripts for proinsulin.

The observation of pancreatic glands free from insulitis under rhIGF-1 treatment, suggests another mechanism that occurs prior the late activation process of infiltrating T cells by eliminating or inactivating the functional properties of autoreactive T cells necessary for beta cell destruction. Recombinant hIGF-1 may exert these effects directly on lymphoid cells, since in vitro suppression of T cell response to concanavalin A or allogeneic stimulation can be achieved in a dose dependent manner (16). Many actions of growth-hormone on the immune system may be mediated by IGF-1 which is also produced by peripheral leukocytes (17). Recent observations suggest that activated T lymphocytes possess receptors for IGF-1 (18-20). In addition, several reports indicate that IGF-1 may influence thymic epithelial cell function in vitro (21) and induce thymocyte replication and differentiation in streptozocin induced diabetic rats (22). Mice which receive 4 mg/kg per day of rhIGF-1 were found to have an increased spleen and thymus weight, due to an increase in the number of lymphocytes in these organs, preferentially T cells from the CD4 phenotype (22). We did not observe any difference in the number of T cells in the lymphoid organs and in the relative contribution of T cell subsets within the spleen, probably because of lower doses of rhIGF-1 used in the present study. Moreover, treatment of diabetic females with rhIGF-1 failed to reduce the capacity of spleen cells to transfer the disease, suggesting that the number and degree of activation of autoreactive T cells were not modified.

The effect may be also on the mechanisms of T cell trafficking into the islets during the 10 days period after T cell inoculation that precedes islet cell invasion (23). T cell homing to the pancreas and endothelial-lymphocyte interactions might be regulatory events. Reconstitution of the thymus of

irradiated congenic NOD-N Thy-1,1 recipients with Thy-1,2$^+$ T cells was not influenced by rhlGF-1. The significant reduction in the number of T cells from donor origin noticed within the spleen may contribute to the protective effects of IGF-1 during adoptive T cell transfer. A reduction in the number but not in the degree of activation of autoreactive T cells may explain why rhlGF-1 treated mice are not fully protected and why diabetes can still occur.

From the present observations, rhlGF-1 should be considered as an important regulator of autoreactive T cells through autocrine but also endocrine actions, which might have clinical consequences during the prediabetic phase in human type 1 Diabetes.

LEGENDS OF FIGURES

Figure 1: Cumulative incidence of diabetes in four independent experiments following adoptive T cell transfer in 24 mice injected twice daily with 10µ rhlGF-1 (open circles) and 21 control mice injected with saline (closed circles).

Figure 2: Severity of insulitis and destructive lesions of recipient mice according to treatment with saline (dark columns) or rhlGF-1 (open columns). Results are mean percentages± SE from 24 individual mice from two independent experiments.

*: $p<0.05$, **: $p<0.01$.

Figure 3: Immunodetection of Thy-1,2$^+$ T cells in the islets of congenic NOD-N Thy-1,1 mice three weeks after adoptive cell transfer of diabetes using 7x106 T cells from NOD Thy-1,2 diabetic donors. Panel A illustrates a severe insulitis in a control mouse. Panel B represents a peri-insulitis in a mouse treated with rhlGF-1

Figure 4: FACS analysis of Thy-1,2$^+$ T cells within the spleen of a congenic NOD-N Thy-1,1 mouse, three weeks after sub-lethal irradiation and inoculation of Thy-1,2$^+$ T cells from diabetic donors. Panel A represents the results in a control NOD-N Thy-1,1 mouse. Insulin like growth factor-1 significantly reduced the number of Thy-1,2$^+$ in the spleen (Panel B) in comparison to saline (Panel C).

Figure 5: FACS analysis of Thy-1,2$^+$ T cells within the thymus of a congenic NOD-N Thy-1,1 mouse, three weeks after sub-lethal irradiation and inoculation of Thy-1,2$^+$ T cells from diabetic donors. Panel A represents the results in a control NOD-N Thy-1,1 mouse. The effects of rhlGF-1 upon the reconstitution of the thymus after T cell transfer are shown in Panel B and are compared to saline injected mouse (Panel C).

TABLE I. Variation in the body weight of experimental mice injected with IGF-1, insulin or saline over a period of 3 weeks. Each value represents the mean±SE (n).

Treatment	Weight (g)		p value
	Day 1	Day 21	
Saline	30.13±0.38 (n=22)	28.71±0.49 (n=21)	0.027
Insulin	29.61±0.39 (n=17)	28.13±0.44 (n=14)	0.018
rhlGF-1	29.63±0.34 (n=24)	29.68±0.52 (n=24)	0.946

TABLE II. Flow cytometry analysis of Thy-1,2$^+$, L3T4$^+$ and Lyt-2$^+$ T cells in the spleens of experimental mice treated with rhlGF-1 or saline. Results are mean ±SE of individual analysis performed on 12 different mice from each group.

Treatment	Percentage of cell population		
	Thy-1,2$^+$	L3T4$^+$	Lyt-2$^+$
Saline	14.78±1.05	14.98±0.76	6.63±0.39
rhlGF-1	21.95±1.87	12.72±.148	5.19±.0.62
p value	0.266	0.258	0.104

TABLE III: Percentages of Thy-1,2$^+$ T cells in the spleen and thymus of experimental congenic NOD-N Thy-1,1 mice three weeks after sub-lethal irradiation and adoptive transfer of 7x10^6 Thy-1,2$^+$ from diabetic donors. Results are the mean±SE of 12 individuals mice.

	Thymocytes		Splenocytes	
	n(x10^6)	% of Thy-1,2$^+$T cells	n(x10^6)	%of Thy-1,2$^+$TCells
rhIGF-1 (n=6)	36.8±2.3	68.43±3.2	60±2.4	10.86±0.5
saline (n=6)	35.1±1.5	72.88±2.5	56±4.8	17.19±1.6
p value	0.559	0.306	0.102	0.004

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CLAIMS

1. Use of IGF-I or analogues thereof in the manufacture of a medicament useful in the prevention of diabetes.

2. Use of IGF-I or analogues thereof in the manufacture of a medicament useful in delaying the clinical onset of diabetes.

3. Use of IGF-I or analogues thereof in the manufacture of a medicament having a protective effect against diabetes.

4. Use of IGF-I or analogues thereof in the manufacture of a medicament preventing beta cell destruction in subjects that are at high risk of developing diabetes.

5. Use of IGF-I or analogues thereof in the manufacture of a medicament for regulating T cells in subjects at high risk of developing diabetes.

6. Method for preventing diabetes by administration of IGF-I or analogues thereof.

7. Method for delaying the clinical onset of diabetes by administration of IGF-I or analogues thereof.

8. Method for protecting against diabetes by administration of IGF-I or analogues thereof.

9. Method for preventing beta cell destruction in subjects which are at high risk of developing diabetes by administration of IGF-I or analogues thereof.

10. Method for regulating of T cells in subjects which are at high risk of developing diabetes by administration of IGF-I or analogues thereof.

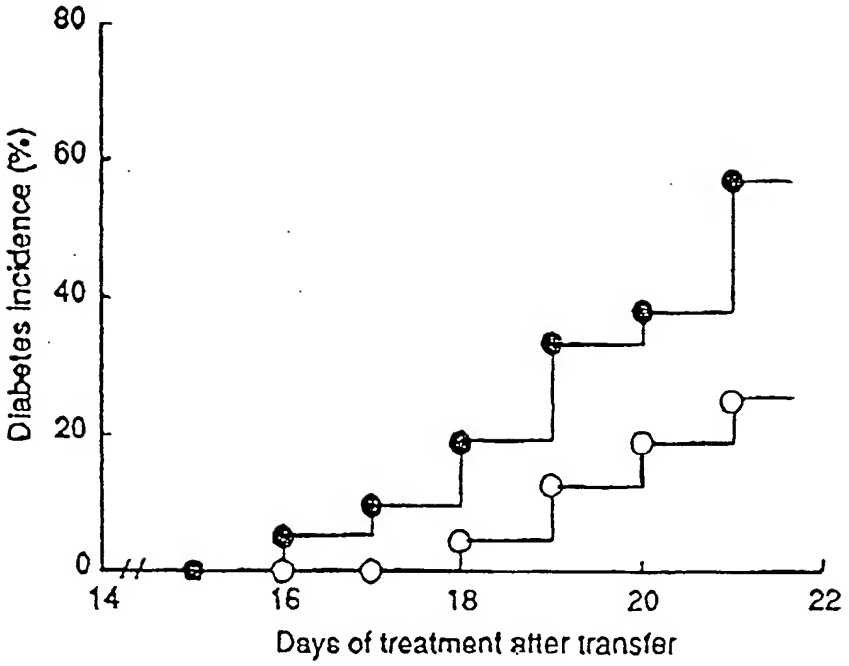
80
60
40
20
0
Diabetes Incidence (%)
14
16
18
20
22
Days of treatment after transfer

Fig. 1

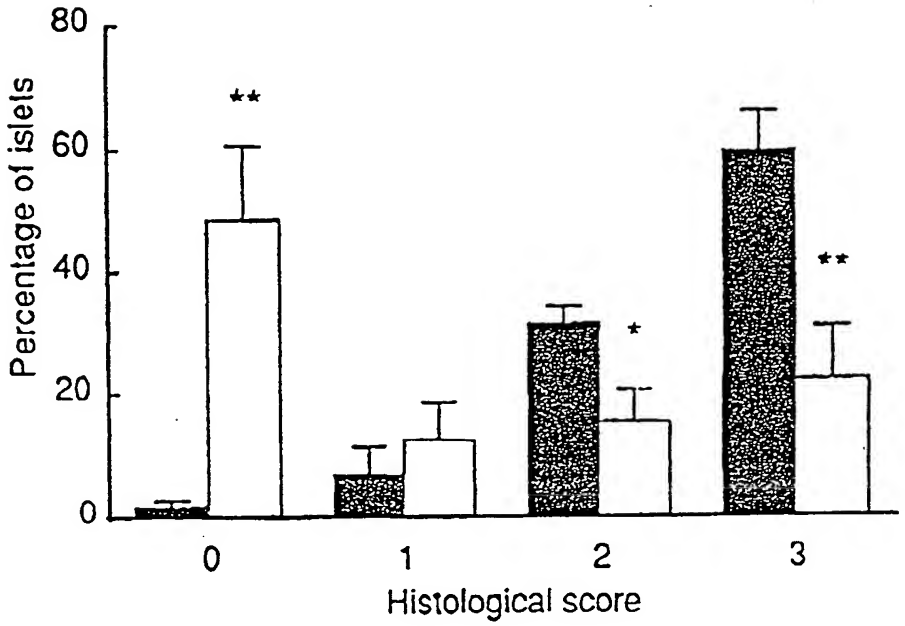

Fig. 2

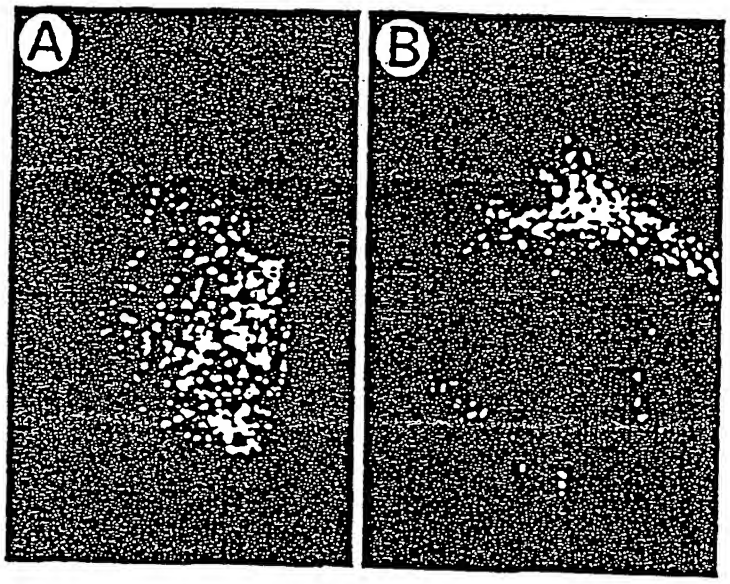

Fig. 3

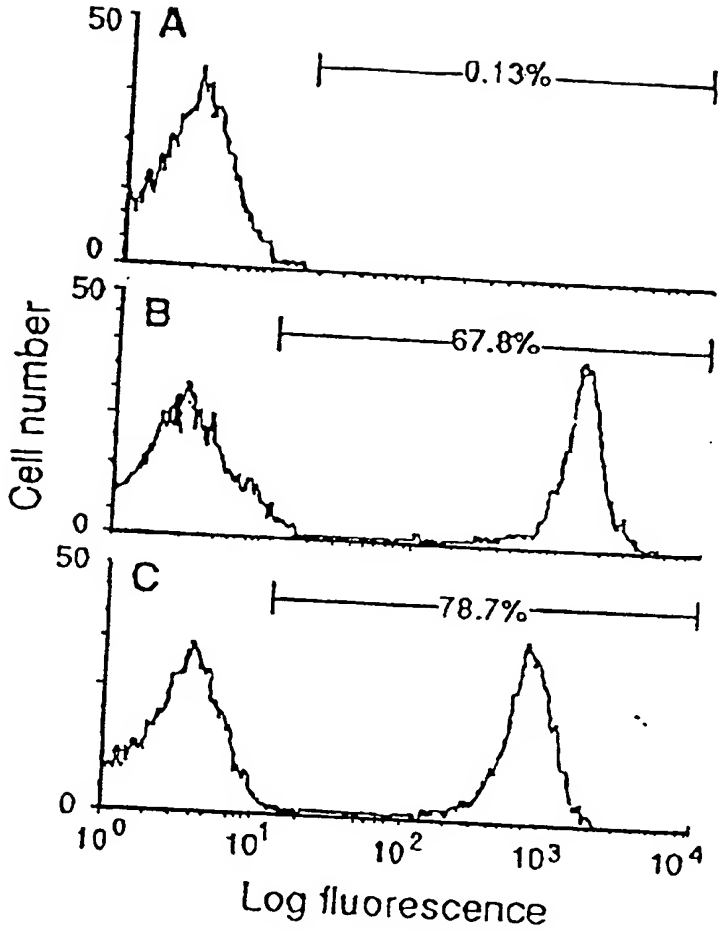
50
A
0.13%
0
50
B
67.8%
0
50
C
78.7%
0
Cell number
10^0
10^1
10^2
10^3
10^4
Log fluorescence

Fig. 4

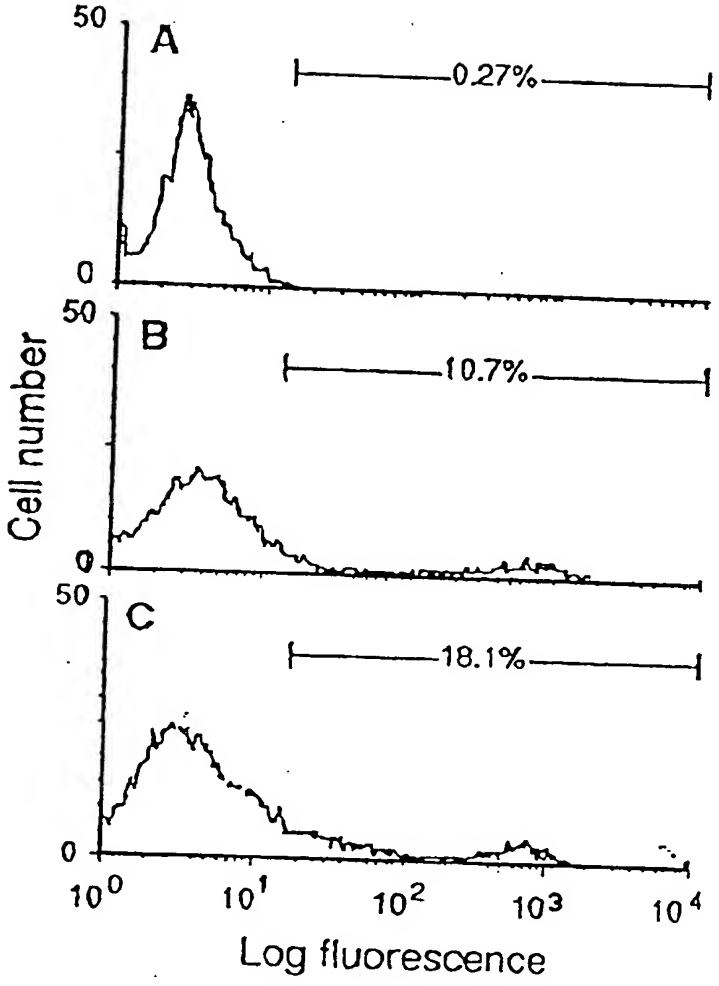
50
A
0.27%
0
50
B
10.7%
0
Cell number
50
C
18.1%
0
10^0
10^1
10^2
10^3
10^4
Log fluorescence

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00776

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/30
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CA, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0331630 A1 (CIBA-GEIGY AG), 6 Sept 1989 (06.09.89) --	1-5
X	Diabetes, Volume 40, April 1991, Luciano Rossetti et al, "Metabolic Effects of IGF-I in Diabetic Rats" page 444 - page 448 --	1-5
X	WO 9323071 A1 (KABI PHARMACIA AB), 25 November 1993 (25.11.93) --	1-5
X	Trends in endocrinology and metabolism,1990, E. Rudolf Froesch et al: "Therapeutic Potential of Insulinlike Growth Factor I", se page 254 --	1-5

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1995	02 -11- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 95/00776

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 6-10
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1.(iv).: Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/10/95

International application No.
PCT/SE 95/00776

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0331630	06/09/89	SE-T3- 0331630	
		AU-A- 2899989	10/08/89
		CA-A- 1336815	29/08/95
		DE-U- 6890520	15/04/93
		DK-B- 169233	19/09/94
		IL-A- 104777	07/10/94
		JP-A- 1233227	19/09/89
		US-A- 4988675	29/01/91
WO-A1- 9323071	25/11/93	NONE	